Int.J.Curr.Microbiol.App.Sci (2017) *6*(7): 3975-3980

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 7 (2017) pp. 3975-3980**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.607.411

Isolation and Screening of Lactic Acid Bacteria for Acidic pH and Bile Tolerance

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Keywords

Lactic acid bacteria, Isolates, Probiotics, Acid tolerance, Bile tolerance.

Article Info

Accepted:
29 June 2017
Available Online:
10 July 2017

A B S T R A C T

As many as 25 lactic acid bacterial strains were isolated from various sources. These isolates were investigated for their tolerance to low pH (3.0, 4.0 and 5.0) and different bile concentrations (0.3 %, 0.4 %, 0.75 % and 1.0 %). Among 25 isolates, strain 42, 45 and 48 showed very high tolerance to acidic pH and the same isolates also tolerated high level of bile salt concentration up to 1.0 per cent. Thus, high resistance to low pH values and to higher concentrations of bile salts enables these strains to survive in the stomach and intestinal environment and to colonize the gut of the host, possess a promising probiotic potential. Hence, these isolates hold potential to be used as probiotics.

Introduction

Lactic acid bacteria (LAB) are Gram positive, non-spore forming, Catalase negative, acid tolerant, fastidious, non-motile and facultative anaerobic friendly gut bacteria. They constitute heterogeneous group of industrially important bacteria. In food industries, they used as preservative, acidulant and flavouring agents by the virtue of their probiotic traits. They are also in use as starter culture in food fermentations such as beverages, yoghurt, vegetables, cereals, meat, cocoa beans etc. (Doyle and Beauchat, 2007). They are also utilized in the development of functional foods and more specifically their application as vaccines, pro and prebiotics, neutraceuticals has attracted new research arena for food scientists and health professionals (Mozzi *et al.*, 2010; De Vuyst *et al.*, 2004).

Traditionally, the fermented foods serve as good sources of new potential sources of LAB. Therefore use of strains of such origin would be of great importance, consumers who suffer from lactose intolerance. In order to exert its beneficial effect on the host, a probiotic strain must be able to survive the gut passage of humans to reach to the action site in viable state and should be in sufficient population (normally 10^7 cells per ml). Thus,

in order to survive in and colonize the gut, the bacteria should express high tolerance to low (acidic) pH and high tolerance to higher concentrations of bile (Kirjavainen *et al.*, 1998). They are supplemented as probiotics that are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance. Hence, as many as 25 isolates of lactic acid bacteria were obtained from various sources such as traditional artisan curds, fermented vegetables, dosa and idli batter, etc. The present work was aimed to evaluate the probiotic potential of these LAB isolates in terms of acid and bile tolerance properties.

Materials and Methods

Isolation of LAB strains, media and culture conditions

As many as 25 LAB strains were isolated from various sources such as traditional fermented arisan curds of Karnataka such as Kohlar, Banashankari, Almatti areas, fermented vegetables etc. by the method of plate count method. The 48 h grown culture was further purified and maintained on de Mann Rogosa Sharpe broth (MRS) (De Man *et al.,* 1960) at -80^0 C by DMSO (cryoprotectant) method.

Test for Acid tolerance

Two ml of overnight grown cultures of the test strains were centrifuged (10 min at 8000 rpm) using centrifuge (HERMLE Z32HK) and the cellular pellet was resuspended in two ml of MRS broth previously adjusted with HCl to different pH levels of 3.0, 4.0 and 5.0. The initial bacterial population soon after inoculation and after 3 h of after incubation was recorded using serial dilution and plate count method on MRS agar. The cultures were incubated at 37^0C in an incubator. The viable counts (CFU/ml) are tabulated.

Test for bile salt tolerance

Similarly, in order to determine the bile tolerance potential of LAB isolates two ml of the overnight grown cultures of the test strains were centrifuged (10 min at 8000 rpm) using centrifuge and the cellular pellet was resuspended in two ml of MRS broth previously adjusted with bile salts (Oxgall Himedia) at different levels such as 0.3 %, 0.4 %, 0.75 % and 1.0 %.

The inoculated cultures were incubated at 37^0 C for 3h. The initial bacterial concentration and after incubation up to 3 h was recorded using optical density spectrophotometer at 650 nm, the counts were tabulated as CFU per ml (Table 4). The control treatment was also maintained and incubated in MRS broth without any bile salts.

Enumeration of viable LAB

Viable cells were enumerated by plating 10-fold dilution on MRS agar medium. The plates were incubated at 37^0C in an incubator for 3 h.

The population was enumerated at initial (immediately after inoculation) and final counts at 90^{th} minute by serial dilution and plate count method at 10^5 dilution. The values obtained were expressed as colony forming units per mililitre (CFU/ml).

Results and Discussion

The evaluation of probiotic potential of the 25 isolates was carried out *in vitro* by stimulating the environment of the gut, namely by subjecting the isolates to low pH values and by exposure to bile salts. The typical transit time of the food in the stomach is between 20 minutes to 3 hours (Hyronimus *et al.,* 2000; Goldin *et al.,* 1992) and therefore the treatment was kept until 3 h time span.

Acid tolerance

Before reaching the intestinal tract, probiotic bacteria must first survive the harsh acidic condition of the stomach. Three pH levels of the growth medium were used where previously adjusted with HCl ranging from 3.0, 4.0 and 5.0 considering the stomach acidity varies from individual to individual (Dunne *et al.*, 2001). The results indicated that among the 25 isolates studied, all showed good tolerance to acid but most of the LAB isolates showed poor growth and population at pH 5.0. The isolates 42, 45 and 48 showed considerably higher at low pH of 3 and 4 compared to pH 5. (Table 1-3). This study confirms that the resistance to the low pH is strain dependent. These results are in confirmation with Mishera *et al.*, (2005), three of their seven isolates *Lactobacillus* sp. tolerated pH 3.0. Although the acid tolerance in lactobacilli is highly strain specific, members of the genus *Lactobacillus* are acidophilic in nature and are able to grow at pH 4.0 in foods containing a fermentable carbohydrate (Alakomi *et al*., 2001).

Table.1 Growth of lactic acid bacterial isolates at pH 3.0

Sl. No.	Code No. of the LAB isolate	*Mean Population (cfu X 10^5 per ml)	
		Initial	After 3 h of incubation
1	**2**	**16.5**	**116.0**
2	**3**	**16.0**	**125.5**
3	**4**	**18.0**	**118.5**
4	**6**	**16.5**	**164.0**
5	**35**	**16.0**	**117.0**
6	**39**	**15.0**	**108.0**
7	**40**	**14.0**	**96.5**
8	**42**	**77.5**	**258.5**
9	**45**	**97.5**	**292.0**
10	**48**	**82.5**	**266.0**
11	**52**	**9.0**	**107.5**
12	**53**	**10.5**	**87.0**
13	**54**	**56.0**	**170.5**
14	**57**	**15.0**	**67.0**
15	**58**	**11.0**	**69.5**
16	**59**	**26.0**	**164.0**
17	**60**	**15.0**	**60.5**
18	**61**	**71.5**	**192.5**
19	**63**	**12.5**	**51.0**
20	**65**	**12.0**	**102.0**
21	**66**	**14.0**	**102.5**
22	**69**	**68.0**	**186.0**
23	**72**	**65.5**	**175.5**
24	**73**	**11.5**	**101.0**
25	**75**	**35.0**	**173.5**

*mean of two replications

Table.2 Growth of lactic acid bacterial isolates at pH 4.0

Sl. No.	Code No. of the LAB isolate	Population (cfu X 10^5 per ml)	
		Initial	After 3 h of incubation
1	**2**	**6.5**	**99.0**
2	**3**	**8.0**	**70.0**
3	**4**	**6.5**	**102.0**
4	**6**	**22.0**	**56.8**
5	**35**	**12.5**	**83.0**
6	**39**	**12.5**	**85.0**
7	**40**	**6.5**	**82.0**
8	**42**	**85.5**	**253.0**
9	**45**	**72.0**	**244.0**
10	**48**	**74.5**	**245.0**
11	**52**	**10.5**	**66.5**
12	**53**	**8.0**	**87.0**
13	**54**	**34.5**	**159.0**
14	**57**	**13.5**	**94.0**
15	**58**	**11.0**	**83.5**
16	**59**	**32.0**	**146.0**
17	**60**	**14.0**	**126.0**
18	**61**	**62.0**	**219.0**
19	**63**	**16.0**	**78.5**
20	**65**	**7.5**	**68.5**
21	**66**	**13.5**	**67.5**
22	**69**	**50.5**	**170.5**
23	**72**	**50.0**	**162.0**
24	**73**	**11.0**	**71.0**
25	**75**	**33.0**	**156.0**

Table.3 Growth of lactic acid bacterial isolates at pH 5.0

Sl No.	Code No. of the LAB isolate	Population (cfu X 10^5 per ml)	
		Initial	After 3 h of incubation
1	**2**	**5.0**	**45.0**
2	**3**	**5.5**	**46.0**
3	**4**	**6.0**	**25.5**
4	**6**	**12.5**	**51.0**
5	**35**	**7.0**	**33.5**
6	**39**	**7.0**	**47.0**
7	**40**	**4.0**	**39.0**
8	**42**	**57.0**	**222.5**
9	**45**	**44.0**	**187.5**
10	**48**	**53.5**	**199.0**
11	**52**	**6.5**	**33.5**
12	**53**	**5.0**	**25.0**
13	**54**	**19.5**	**130.0**
14	**57**	**4.0**	**41.5**

15	**58**	**5.5**	**41.0**
16	**59**	**17.0**	**99.0**
17	**60**	**6.0**	**35.0**
18	**61**	**42.0**	**176.5**
19	**63**	**5.5**	**41.0**
20	**65**	**6.5**	**42.0**
21	**66**	**6.5**	**45.0**
22	**69**	**34.0**	**156.0**
23	**72**	**22.5**	**147.0**
24	**73**	**6.0**	**36.0**
25	**75**	**22.5**	**121.5**

Table.4 Growth of lactic acid bacterial isolates at different bile concentrations (OD values)

LAB ISOLATE	0.3 %		0.4 %		0.75 %		1.0 %	
	0:0 h	03:00 h	0:0 h	03:00 h	0:0 h	03:00 h	0:0 h	03:00 h
2	**0.120**	**0.225**	**0.117**	**0.680**	**0.123**	**0.225**	**0.122**	**0.256**
3	**0.085**	**0.215**	**0.090**	**0.796**	**0.075**	**0.081**	**0.085**	**0.092**
4	**0.071**	**0.265**	**0.128**	**0.450**	**0.114**	**0.116**	**0.131**	**0.203**
6	**0.053**	**0.033**	**0.069**	**0.066**	**0.090**	**0.590**	**0.090**	**0.619**
35	**0.130**	**0.189**	**0.112**	**0.769**	**0.108**	**0.239**	**0.123**	**0.274**
39	**0.095**	**0.505**	**0.074**	**0.236**	**0.084**	**0.110**	**0.094**	**0.121**
40	**0.076**	**0.109**	**0.065**	**0.749**	**0.063**	**0.084**	**0.080**	**0.100**
42	**0.097**	**1.115**	**0.120**	**1.093**	**0.133**	**1.099**	**0.135**	**1.041**
45	**0.083**	**0.070**	**0.111**	**0.080**	**0.120**	**0.139**	**0.119**	**0.933**
48	**0.110**	**0.122**	**0.125**	**1.175**	**0.135**	**1.195**	**0.190**	**1.273**
52	**0.078**	**0.103**	**0.053**	**0.840**	**0.053**	**0.080**	**0.048**	**0.086**
53	**0.080**	**0.097**	**0.082**	**1.060**	**0.086**	**0.165**	**0.090**	**0.199**
54	**0.037**	**0.031**	**0.069**	**0.069**	**0.091**	**0.332**	**0.092**	**0.600**
57	**0.054**	**0.079**	**0.022**	**0.841**	**0.053**	**0.091**	**0.058**	**0.116**
58	**0.059**	**0.066**	**0.058**	**0.871**	**0.056**	**0.075**	**0.056**	**0.073**
59	**0.051**	**0.053**	**0.071**	**0.071**	**0.090**	**0.631**	**0.091**	**0.713**
60	**0.090**	**0.145**	**0.109**	**0.603**	**0.109**	**0.178**	**0.071**	**0.093**
61	**0.063**	**0.055**	**0.082**	**0.090**	**0.100**	**0.617**	**0.120**	**0.831**
63	**0.150**	**0.171**	**0.110**	**0.756**	**0.061**	**0.090**	**0.062**	**0.078**
65	**0.100**	**0.121**	**0.069**	**0.862**	**0.058**	**0.127**	**0.049**	**0.119**
66	**0.098**	**0.251**	**0.073**	**0.838**	**0.071**	**0.108**	**0.074**	**0.125**
69	**0.081**	**1.093**	**0.120**	**1.095**	**0.130**	**0.139**	**0.120**	**0.947**
72	**0.080**	**0.062**	**0.090**	**0.059**	**0.115**	**0.851**	**0.131**	**0.854**
73	**0.098**	**0.237**	**0.110**	**0.719**	**0.098**	**0.148**	**0.095**	**0.149**
75	**0.050**	**0.055**	**0.077**	**0.081**	**0.092**	**0.615**	**0.120**	**0.750**

The probiotic effect of LAB may partly be based on the production of relevant concentrations of lactic acid in the microenvironment, which in combination with bile salts, inhibits the growths of Gram negative pathogenic bacteria (Stiles and Holzapfel, 1997).

Bile tolerance

Bile salts belong to the factors that may significantly affect the viability of LAB in the GIT, influencing the health of the host. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of the

bacteria in the small intestine of the host (Havenaar *et al.,* 1992). This will help LAB to reach the small intestine and colon and contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010).

After 3 h of incubation in MRS medium supplemented with 0.3% bile all isolates showed variable rates of growth (Table 3). All the isolates showed good survivability at lower concentrations (0.3 %, 0.4 %). However, few isolates such as 42, 45 and 48 survived even to the higher bile concentrations (0.75 % and 1.0 % oxgall). Thus, these results indicate that these isolate could be used as potential candidates as probiotics (Table 3).

In conclusion, the LAB isolates used in the study showed a high resistance to low pH levels and to higher bile salt concentrations. These traits may enable them to survive in the stomach and intestine or even to compete with other bacterial groups in this environment and to colonize the gut of the host. The results indicated that isolates 42, 45 and 48 showed very high tolerance to acidic pH as well as to higher bile salt concentrations. Hence, the results indicating that their potential to be explored as probiotic strains in future.

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How to cite this article:

Noor Nawaz, A.S., K.S. Jagadeesh and Krishnaraj, P.U. 2017. Isolation and Screening of Lactic Acid Bacteria for Acidic pH and Bile Tolerance. *Int.J.Curr.Microbiol.App.Sci.* 6(7): 3975-3980. doi: https://doi.org/10.20546/ijcmas.2017.607.411